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| Applicant: | OKOCHI et al. | Examiner | BUNNER, Bridget E. |
| Serial No.: | 10/521691 | Group Art: | 1647 |
| Filed: | August 31, 2005 | Docket: | 10873.1604USWO |
| Title: | NOVEL NOTCH-ORIGIN POLYPEPTIDES AND BIOMARKERS AND REAGENTS USING THE SAME | | |

CERTIFICATE UNDER 37 CFR 1.10
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By: _____
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By:
Name: Stephanie Samuell

## COMMUNICATION REGARDING PRIORITY DOCUMENT

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Dear Sir:

Further to the Response to Restriction Requirement filed March 26, 2008, we enclose a verified translation of the priority application. The priority date of the present application is prior to the October 2002 publication date of the Okochi et al. reference cited in the Restriction Requirement. Please charge any additional fees or credit overpayment to Deposit Account No. 50-3478.

Respectfully submitted,



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Dated: June 18, 2008

By:
Douglas P. Mueller
Reg. No. 30,300
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# VERIFICATION OF TRANSLATION

U.S. Patent Application No. 10/521,691

Filing Date: 31 August, 2005

Title of the Invention: NOVEL Notch-ORIGIN POLYPEPTIDES AND BIOMARKERS AND REAGENTS USING THE SAME

I, Masako TAKESHITA, whose full post office address is IKEUCHI・SATO & PARTNER PATENT ATTORNEYS, 26th Floor, 8-30, Tenmabashi 1-chome, Kita-ku, Osaka-shi, Osaka 530-6026, JAPAN

am the translator of the document attached and I state that the following is a true translation to the best of my knowledge and belief of Japanese Patent Application No. JP2002-210040 (Date of Application: 18 July 2002).

At Osaka, Japan

Dated this 27/ 5/ 2008 (Day/Month/Year)

Signature of translator:

Masako Takeshita

Masako TAKESHITA

PATENT OFFICE

JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application: July 18, 2002

Application Number: Patent Application No. JP 2002-210040

Applicant(s): Osaka Industrial Promotion Organization

[Document Name] Patent Application

[Case Number] R6659

[Date of Application] July 18, 2002

[Destination] Commissioner of the Japanese Patent Office

[International Patent Classification] C07C239/14

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[Phone Number] 06-6135-6051

[Official Fee]

[Advance Payment Note Number] 139757

[Amount of Payment] 21000

[List of File Documents]

| | | |
|---|---|---|
| [Name of Document] | Specification | 1 |
| [Name of Document] | Drawings | 1 |
| [Name of Document] | Abstract | 1 |

[Proof] Required

[Document Name] SPECIFICATION

[Title of the Invention] NOVEL Notch-ORIGIN POLYPEPTIDES AND BIOMARKERS AND REAGENTS USING THE SAME

[Claims]

[Claim 1] A novel polypeptide derived from a Notch protein, wherein in a series of proteolytic events of the Notch protein, the polypeptide is released to an extracellular space when NICD (Notch intracellular cytoplasmic domain) translocates to a nucleus as a result of intramembranous endoproteolysis that occurs subsequent to extracellular proteolysis.

[Claim 2] The polypeptide according to claim 1, which is released to the extracellular space in proportion to Notch signal transduction.

[Claim 3] The polypeptide according to claim 1 or 2, wherein the release of the polypeptide to the extracellular space results from presenilin-dependent proteolysis.

[Claim 4] The polypeptide according to any one of claims 1 to 3, which is produced and released as a result of proteolysis (S4 cleavage) of the Notch protein that occurs simultaneously with or either before or after proteolysis at S3, the proteolysis (S4 cleavage) occurring on a N-terminal side with respect to the S3 in a transmembrane domain of the Notch protein.

[Claim 5] The polypeptide according to claim 4, wherein the cleavage site (S4) on the N-terminal side with respect to the S3 is an amino acid residue in the transmembrane domain.

[Claim 6] A polypeptide comprising an amino acid sequence of at least one of SEQ ID NOS: 1 to 18.

[Claim 7] A polypeptide comprising an amino acid sequence of at

least one of SEQ ID NOS: 1 to 18 in which one or several of amino acids are deleted, substituted, or inserted, wherein the polypeptide is derived from a Notch protein, and in a series of proteolytic events of the Notch protein, the polypeptide is released to an extracellular space when NICD translocates to a nucleus as a result of intramembranous endoproteolysis that occurs subsequent to extracellular proteolysis.

[Claim 8] The polypeptide according to claim 7, which is released to the extracellular space in proportion to a Notch signal.

[Claim 9] The polypeptide according to claim 7 or 8, wherein the release of the polypeptide to the extracellular space results from presenilin-dependent proteolysis.

[Claim 10] A biomarker comprising the polypeptide according to any one of claims 1 to 9.

[Claim 11] The biomarker according to claim 10 for detecting at least one selected from the group consisting of Notch signal transduction, cell differentiation, tumor, apoptosis, and Alzheimer's disease.

[Claim 12] An antibody that can recognize the polypeptide according to any one of claims 1 to 9.

[Claim 13] The antibody according to claim 12, which is a monoclonal antibody or a polyclonal antibody.

[Claim 14] A reagent for detecting at least one selected from the group consisting of Notch signal transduction, cell differentiation, tumor, apoptosis, and Alzheimer's disease, which comprises the antibody according to claim 12 or 13.

[Claim 15] A gene encoding the polypeptide according to any one of claims 1 to 9.

[Claim 16] The gene according to claim 15, which is DNA or RNA.

[Claim 17] A vector comprising the gene according to claim 15 or 16.

[Claim 18] A transformant transformed with the vector according to claim 17.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to novel polypeptides derived from novel intramembranous endoproteolysis of Notch proteins (hereinafter also referred to collectively as "Notch") and to biomarkers and reagents using the same. In the description of the present invention, the following abbreviations are used for cleavage sites of Notch: S1 for Site-1, S2 for Site-2, S3 for Site-3, and S4 for Site-4. As will be described later, Site-4 (S4) is a novel intramembranous cleavage site discovered by the inventors of the present invention.

[0002]

[Prior Art]

Notch is a type I transmembrane protein present on a cell surface. It contains a repeated EGF-like domain in its extracellular domain and NICD (Notch Intracellular Cytoplasmic Domain), which is a transcription factor containing an ankyrin repeated domain, in its intracellular domain. It has been known that Notch plays a role in intercellular signaling relating to cell differentiation. For example, in the developmental process of a cranial nerve system, some of the cells derived from ectoderm differentiate into neuronal precursor cells (stem cells) and further into nerve cells or glial cells, during which intercellular signaling via Notch is important. The mechanism of the intercellular signaling via Notch is as follows. First, Notch is expressed as a receptor on a Notch signal-receiving cell. During

the transport to the cell surface, the Notch undergoes the cleavage at the extracellular domain (S1) by a protease such as furin, and the two Notch fragments resulting from the S1 cleavage are held together through an S-S bond on the cell surface. Next, when a Notch signal-sending cell is present near the Notch signal-receiving cell, a Notch ligand (e.g., Delta, Serrate, or Lag-2, belonging to a DSL family) is expressed on the surface of the Notch signal-sending cell. Under these two conditions, the Notch ligand interacts with the Notch receptor on the cell surface, whereby sequential proteolytic events are induced to trigger signal transduction. More specifically, the Notch is cleaved at a site (S2) close to the cell membrane surface, which triggers the cleavage at a site (S3) that is either inside the cell membrane or in close proximity to the cell membrane inside the cell. NICD, which is the intracellular domain of the Notch resulting from the S3 cleavage, is released to an intracellular space and translocates to the nucleus, where it binds to a CSL family (CPB, SuH, or Lag-1; transcription factor) to regulate the transcription of target genes. Presenilin, which is associated with Alzheimer's disease, is involved in the S3 cleavage.

[0003]

As described above, Notch plays an extremely important role in intercellular signaling for cell differentiation. Moreover, recent studies have revealed that Notch is involved not only in the differentiation of a cranial nerve system as described above but also in cell tumorigenesis, apoptosis, Alzheimer's disease, etc., which causes Notch to become a focus of attention (see Okochi *et al.*, "Biology of Alzheimer's disease and presenilin", Bunshi Seishin Igaku, Vol. 1, No. 3, 2001; Kageyama *et al.*, "Notch pathway in neural development", Tanpakushitsu Kakusan Koso, Vol. 45, No. 3, 2000; and Brian *et al.*, "A carboxy-terminal deletion mutant of Notch 1 accelerates

lymphoid oncogenesis in E2A-PBX1 transgenic mice", Blood, Vol. 96, No.5, 2000 Sep.1, pp 1906-1913). Therefore, the detection of Notch signal transduction is extremely important for research and diagnosis of cell differentiation, cell tumorigensis, apoptosis, Alzheimer's disease, etc., and the earlier possible establishment of the technology for detecting Notch signal transduction is being demanded.

[0004]

[Problems to be Solved by the Invention]

Therefore, with the foregoing in mind, it is an object of the present invention to provide a substance that can serve as an extracellular secreted marker for detecting Notch signal transduction.

[0005]

[Means for Solving the Problems]

The inventors of the present invention hypothesized that, during a series of proteolytic events of Notch, a polypeptide remaining in a cell membrane is released to an extracellular space as a result of the cleavage occurring at S3, and decided to examine this hypothesis. This is because, if the polypeptide remaining in the cell membrane is released to an extracellular space, it can serve as a marker for Notch signal transduction. Through a series of studies on Notch signal transduction, the inventors of the present invention found out that a fourth cleavage occurs at a site (in the transmembrane domain) different from the S3 cleavage site and a polypeptide resulting from this fourth cleavage is released to an extracellular space. Based on this finding, the inventors arrived at the present invention.

[0006]

That is, the novel polypeptide according to the present invention is a

novel polypeptide derived from a Notch protein. In a series of proteolytic events of the Notch protein, the polypeptide is released to an extracellular space when NICD (Notch intracellular cytoplasmic domain) translocates to a nucleus as a result of the intramembranous endoproteolysis that occurs subsequent to the extracellular proteolysis. This polypeptide can be detected by using an antibody or the like, and thus can be used as a marker for detecting Notch signal transduction. Furthermore, since Notch signal transduction is involved in cell differentiation, cell tumorigensis, Alzheimer's disease, apoptosis, etc., the novel polypeptide according to the present invention also can be used as a marker for detecting them. Moreover, as will be described later, there are several types of novel polypeptide according to the present invention with their C-termini being different from each other. Hereinafter, the novel polypeptide according to the present invention is referred to also as "Notch-β (Nβ)". Also, the above-described intramembranous endoproteolysis is not limited to that occurring in a cell membrane but includes that occurring in an organelle membrane of a cell.

[0007]

[Embodiments]

Hereinafter, the present invention will be described further in detail.

[0008]

A polypeptide according to the present invention is released to an extracellular space in proportion to Notch signal transduction. Besides, novel proteolysis that occurs immediately before the release of the polypeptide to the extracellular space is presenilin dependent, and inhibition of the presenilin function causes a decrease in the release of the polypeptide of the present invention.

[0009]

The novel polypeptide according to the present invention is produced and released as a result of the proteolysis (S4 cleavage) of a Notch protein that occurs simultaneously with or either before or after the proteolysis of the Notch protein at S3. The proteolysis (S4 cleavage) occurs on a N-terminal side with respect to S3 in a transmembrane domain of the Notch protein.

[0010]

The novel polypeptide (Nβ) according to the present invention is a polypeptide including an amino acid sequence selected from SEQ ID NOS: 1 to 18. In these SEQ ID NOS: 1 to 18, SEQ ID NOS: 1 to 9 represent murine amino acid sequences, while SEQ ID NOS: 10 to 18 represent human amino acid sequences. In the amino acid sequences represented by the SEQ ID NOS: 1 to 18, one or several of the amino acids may be deleted, substituted, or inserted. Polypeptides represented by such amino acid sequences also are derived from Notch proteins, and are released to an extracellular space when NICD translocates to a nucleus as a result of intramembranous endoproteolysis that occurs subsequent to extracellular proteolysis in a series of proteolytic events of the Notch proteins. These polypeptides also are released to an extracellular space in proportion to a Notch signal in a presenilin-dependent manner. It is to be noted that the novel polypeptide according to the present invention may be derived from a living organism or may be synthesized artificially. The living organism is not limited to a particular type, and may be, for instance, a human, a mouse, a rat, a rabbit, a goat, a swine, a bovine, a drosophila, or a nematode. Also, the type of tissue or cell from which the novel polypeptide of the present invention is derived is not particularly limited. More specifically, somatic

cells and tissues, such as nerve, marrow, and cancer cells and tissues, may be the source of the polypeptide of the present invention, regardless of whether undifferentiated or differentiated.

[0011]

A biomarker according to the present invention contains the above-described novel polypeptide of the present invention. The biomarker of the present invention can be used for detecting Notch signal transduction, cell differentiation, tumor, apoptosis, Alzheimer's disease, or the like. The biomarker of the present invention further may contain other components, or alternatively, it may be the novel polypeptide itself (i.e., the biomarker may contain the novel polypeptide alone). This biomarker can be detected using a reagent containing an antibody that can recognize the novel polypeptide. The antibody that can recognize the novel polypeptide can be prepared by an ordinary method, and may be a monoclonal antibody or a polyclonal antibody. In addition to the antibody that can recognize the novel polypeptide, the reagent further may contain a labeled antibody against this antibody or a labeled antibody that can recognize the novel polypeptide. The labeling can be achieved, for example, by using a fluorescent substance, an enzyme (e.g., an enzyme that acts on a substrate that develops color when reacting with the enzyme), a radioactive substance, or a carrier such as agarose.

[0012]

A gene according to the present invention is a gene encoding the novel polypeptide of the present invention, and may be DNA or RNA. A vector according to the present invention is a vector containing the above-described gene, and a transformant according to the present invention is a transformant transformed with the above-described vector.

[0013]

Next, an example of the extracellular release of the novel polypeptide according to the present invention will be described with reference to the left region of FIG. 7. It is to be noted that the right region of FIG. 7 shows an example of the extracellular release of amyloid-β (Aβ) in Alzheimer's disease. As shown in the left region of FIG. 7, the amino terminus of NEXT (Notch Extracellular Truncation) is produced as a result of extracellular cleavage by TACE (TNFa-Converting Enzyme). The NEXT resulting from the S2 cleavage then undergoes S3 cleavage, and NICD resulting from the S3 cleavage translocates to the nucleus. Cleavage at S4 (the fourth cleavage site of Notch newly discovered by the inventors of the present invention) occurs simultaneously with or either before or after the S3 cleavage, so that Nβ (a novel polypeptide according to the present invention) is released to an extracellular space.

[0014]

Next, an example of C-terminus amino acid sequences of novel polypeptides (Nβs) of the present invention will be described with reference to FIG. 4b. FIG. 4b shows sequences near the C-termini of Nβs or fragments released to an extracellular space with regard to 4 types of murine Notch (mNotch1 to mNotch4), 4 types of human Notch (hNotch1 to hNotch4), and βAPP. As shown in FIG. 4b, the major S4 cleavage site resides a few amino acid residues closer to the N-terminus with respect to the center of putative transmembrane domain (TM) (indicated by the triangular arrowhead on the left in the drawing). Furthermore, as shown in FIG. 4b, amino acid sequences around the major cleavage site are not conserved in mNotch-1 to mNotch-4, though valine 1743 as the S3 cleavage site is conserved (indicated by the triangular arrowhead on the right in the

drawing). Thus, the S4 cleavage site is characterized by its diversity, unlike the S3 cleavage. It is speculated that this diversity might reflect the peculiarity of the mechanism by which S4 secretase recognizes the sequence of the cleavage site.

[0015]

[EXAMPLES]

Hereinafter, the present invention will be described by way of examples. Reagents, materials, and experimental procedures used in the respective examples are as follows.

[0016]

(Reagent)

A γ-Secretase inhibitor, [(2R, 4R, 5S)-2-Benzyl-5-(Boc-amino)-4-hydroxy-6-phenyl-hexanoyl]-Leu-Phe-NH2, was purchased from Bachem.

[0017]

(Plasmids)

cDNAs encoding Notch ΔE-M1727V (NΔE) and NICD with C-terminal 6 x c-myc tag inserted in pcDNA3 hygro were prepared in the manner described in Schroeter *et al.* (Schroeter, E.H., Kisslinger, J.A., Kopan, R. (1998), "Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain", Nature, 393, 382-386). The cDNAs were gift from Dr. R. Kopan. N-terminally FLAG-tagged NEXT, i.e., FLAG-NEXT (F-NEXT), was prepared by 2-step site-directed mutagenesis. In the first step, F-NEXT M1727V was produced using the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene). NΔE was used as a template, and the following two primers 1 and 2 (SEQ ID NO: 19 and SEQ ID NO: 20) were prepared.

[0018]

Primer 1:

5-P-ATCGTCGTCCTTGTAGTCTCTCAAGCCTCTTGCGCCGAGCGCGGGC AGCAGCGTTAG-3'

Primer 2:

5-P-GACAAGATGGTGATGAAGAGTGAGCCGGTGGAGCCTCCGCTGCCC TCGCAGCTG-3'

[0019]

In the second step, F-NEXT was prepared by site-directed mutagenesis using Quick Change Site-Directed Mutagenesis Kit (Stratagene). The F-NEXT M1727V was used as a template, and the following two primers 3 and 4 (SEQ ID NO: 21 and SEQ ID NO: 22) were prepared.

[0020]

Primer 3: 5-CCTCGCAGCTGCACCTCATGTACGTGGCAGCG-3'

Primer 4: 5-CGCTGCCACGTACATGAGGTGCAGCTGCGAGG-3'

[0021]

Each mutant was sequenced to verify successful mutagenesis.

[0022]

(Antibodies)

The polyclonal antibody (L652) is an antibody against a polypeptide with the amino acid sequence from V 1722 to G 1743 of human Notch1 (i.e., the sequence between S2 and S3). The antibody (L652) was produced in the following manner. First, the above-described polypeptide serving as an antigen was provided. This polypeptide is characterized in that it contains a lot of hydrophobic amino acids. On this account, the antibody was produced in the same manner as that used for producing an antibody against the Alzheimer's disease amyloid β-protein. More specifically, the

antibody was produced in the following manner. The polypeptide was dissolved in water directly without being conjugated with any carrier protein. After addition of the same volume of 2 × phosphate buffer, the polypeptide was emulsified with adjuvant and injected into rabbits (Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., Haass, C. (1997), "Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42", J Biol Chem 272, 16085-16088). A monoclonal antibody (9E10) against c-myc and a reagent (M2-agarose) in which a monoclonal antibody against FLAG is covalently bound to agarose were commercially available.

[0023]

(Cell cultures and cell lines)

Human embryonic kidney 293 (K293), N2a and COS cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 200 μg/ml zeocin (to select for PS1 expression), and/or 100 μg/ml hygromycin (to select for NΔE and F-NEXT expression). The K293 can stably express PS1 wt, PS1 L286V, or PS1 D385N, ADDIN ENRfu (Okochi *et al.*, 2000, Kulic *et al.*, 2000, Wolfe *et al.*, 1999). The transfection with NΔE or F-NEXT was performed by means of a product named Lipofectamine 2000 (Invitrogen).

[0024]

(Pulse-chase)

To determine NΔE N-terminal fragment (NTF: Nβ) release from NΔE expressing cells, K293 cells stably transfected with NΔE or NICD were grown to confluence in a 10 cm dish. The cells were then metabolically pulse-labeled for 2 hours with 300 μCi ($^{3}H$) amino acids (tritiated amino acid mixture, Amersham) in Earle's Balanced Salt Solution supplemented with

MEM Vitamine Solution (Gibco) and several cold amino acids, followed by a 6-hour chase by 10% FCS/DMEM. To examine Nβ release, cells expressing F-NEXT were, at first, starved of methinine for 40 min with methionine-free media and then pulse-labeled for 1 hour with 400 μCi ($^{35}S$) amino acid mixture (Redivue Promix, Amersham) in methionine-free DMEM, followed by chasing for various time periods with the chase media containing 10% FCS/DMEM supplemented with excess cold methionine.

[0025]

(Immunoprecipitation/SDS-PAGE)

At the end of the respective chase periods, the media were collected and put on ice immediately, followed by centrifugation at 3000 × g to exclude cell debris. Next, a protease inhibitor cocktail (1:1000; Sigma) and 0.025% of sodium azide were added. The thus-obtained samples were immunoprecipitated with L652 or M2-agarose (Sigma) overnight and then washed three times with RIPA buffer containing 0.1% SDS, 0.5% deoxycholic acid, and 1% TritonX-100, followed by SDS-PAGE using Tris-Tricine 10% to 20% gradient gel (Invitrogen). The cells were scraped in ice-cold PBS, and then harvested by means of 1500 × g centrifugation, followed by lysation with 100 μl of 10 × RIPA. 900 μl of PBS with a protease inhibitor mix (1:500; Sigma) was then added to the lysed cells. The insoluble fraction was separated by 15000 × g centrifugation and the resultant supernatant was used for immunoprecipitation. The samples for immunoprecipitation were pretreated by protein A sepharose (Sigma) and immunoprecipitated with 9E10 or M2 agarose. Next, the washed protein samples were separated by 8% or Tris-Tricine SDS-PAGE. After fixation, the gel was shaken in Amplify Fluorographic Reagent (Amersham), dried, and autoradiographed.

[0026]

(Immunoprecipitation /MALDI-TOF MS analysis)

After cells stably expressing the F-NEXT and their derivatives were grown to confluence in a 20 cm dish, the culture media were replaced with fresh 10% FCS/DMEM. After the cells with the fresh conditioned media were cultured for 3 hours in a $CO_2$ incubator, the culture media were collected and immediately put on ice and centrifuged to eliminate cell debris. After supplementation with a protease inhibitor mix (1:1000) and 0.025% sodium azide, the media were immunoprecipitated with M2 agarose for 4 hours at 4°C. The samples were then washed three times for 10 min at 4°C with an MS wash buffer containing 0.1% n-octylglucoside, 140 mM NaCl, 10 mM Tris (pH 8.0), and 0.025% sodium azide. The samples were then washed once again with 10 mM Tris (pH 8.0) containing 0.025% sodium azide. Peptides bound to the resultant precipitates were eluted with TFA/Acetonitrile/Water (TFA :acetonitorile:water = 1:20:20) saturated with α-cyano-4 hydroxy cinnamic acid. The solubilized samples were dried on a stainless plate and subjected to a MALDI-TOF MS analysis. MS peaks were calibrated using angiotensin (Sigma) and insulin β-chain (Sigma).

[0027]

(Example 1)

Detection of N-terminal fragment (NTF; F-Nβ) of FLAG-NEXT (F-NEXT) in culture media

FIG. 1a is a schematic illustration of structures of NΔE, F-NEXT, and NICD. As shown in FIG. 1a, in F-NEXT, a signal peptide and also a FLAG sequence and two methionines subsequent to the signal peptide are inserted into the N-terminus of NEXT. The 1727th amino acid residue was not mutated in the F-NEXT. However, in NΔE (murine Notch1 (mNotch-1)),

methionine was artificially mutated to valine, as indicated by the inverse triangle in FIG. 1a (Schroeter, E.H., Kisslinger, J.A., Kopan, R. (1998), "Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain", Nature, *393*, 382-386.). The arrowhead indicates a S3 cleavage site.

[0028]

Cells stably expressing NΔE or F-NEXT were pulse-labeled for 1 hour with ($^{35}$S) and chased for the time period indicated in FIG. 1b. The resultant cell lysates were immunoprecipitated with 9E10 and analyzed by 8% SDS-PAGE. As shown in FIG. 1b, proteolysis of NΔE (a left panel) and F-NEXT (a right panel) was observed after a 2-hour chase, which resulted in NICD bands migrating faster than those of NΔE and F-NEXT. With regard to the NICD production efficiency, there was no difference between the cells expressing NΔE and the cells expressing F-NEXT.

[0029]

On the one hand, the culture media were immunoprecipitated with M2 agarose. As shown in the lower panel of FIG. 1b, a band of F-Nβs (an aggregate of novel polypeptide groups according to the present invention) of about 4 kDa was identified only in the 2-hour chased media of the cells stably expressing F-NEXT. The result indicates an entirely new finding that, during the NICD production, an amino terminal fragment on the side opposite to the NICD is secreted into an extracellular space.

[0030]

F-NEXT expressing cells were pulse-labeled with ($^{35}$S) for 1 hour and chased for the time periods indicated in FIG. 1c. F-Nβs in the media and the lysates were examined by the above-described experimental procedures. As shown FIG. 1d, accumulation of F-Nβs (an aggregate of novel polypeptide

groups according to the present invention) in accordance with the extension of chase period was observed in the media, but was hardly detectable in the cell lysates. However, with longer exposure of a film when taking a picture of electrophoresis gel, a F-Nβ band with the same molecular weight (hereinafter referred to as "MW") as in the media was also detectable in the lysates (data not shown).

[0031]

The results shown in FIGs. 1b and 1d were reproduced when F-NEXT M1727V mutant was used or when CHO, COS, and N2a were used as the expressing cells (data not shown).

[0032]

(Example 2)

Detection of N-terminal fragment (NTF: Nβ) of NΔE in culture media

K293 cells stably expressing NΔE or NICD were pulse-labeled with ($^{3}$H) for 2 hours and chased for 6 hours. Chased media and cell lysates were immunoprecipitated with L652, an antibody against N, and the thus-obtained samples were separated by Tris-Tricine SDS-PAGE. As shown in FIG. 2a, a NΔE NTF band (indicated by the arrowhead) of MW 3 to 4 kDa was detected in the culture media of the NΔE cells, but not from which the NICD is expressed. The band was not identified to be produced in the cell lysates. Thus, it was considered that the band was of wild-type Nb that were not FLAG-tagged.

[0033]

The same media and lysates as in the above were immunoprecipitated with an anti-myc antibody (9E10). As shown in the lower panel of in FIG. 2(b), about 100 kDa bands of NΔE and NICD were detected in the lysates but not in the media. This result suggests that NΔE

and NICD were expressed in the respective cells at substantially the same rate.

[0034]

(Example 3)

Identification of C-termini of Nβs released to culture media

FIG. 3(b) is a schematic illustration of intramembranous cleavage of murine Notch-1 (mNotch1) and human βAPP. As a result of the intramembranous cleavage of mNotch1, NICD and Nβ are produced. In the present example, Nβ secretion and a novel cleavage site at the C-terminus of Nβ were confirmed. On the other hand, as a result of the intramembranous cleavage of βAPP, an intracellular fragment CTFγ50 (Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M.M., Teplow, D.B., Haass, C. (2001), "Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch", EMBO Rep. *2*, 835-841.) and several types of Aβ fragments are produced.

[0035]

Culture media of cells stably expressing F-NEXT were immunoprecipitated with M2 agarose, and MW of Nβs were analyzed by means of MALDI TOF/MS according to the above-described experimental procedures. The result is shown in the graph (large) shown in FIG. 3a. As shown in the graph, multiple peaks were observed around MW 4000, but no significant peaks of MW more than 4500 were identified. The graph (small) shown in FIG. 3a shows the details of the peaks from MW 3000 to 4500. The same major peaks were identified when CHO, COS and N2a were used as host cells (data not shown). These peaks also were identified when transfected with F-NEXT M1727V mutant (data not shown).

[0036]

FIG. 4a shows a list of amino acid sequences of Nβs corresponding to the MALDI-TOF MS peaks shown in the FIG. 4b. The C-terminus of the major Nβ was alanine 1731. Bold letters indicate an amino acid sequence of the major peak. As shown in FIG. 4(a), no peaks of MW around 5060, corresponding to a S3 cleavage site, were identified. From these results, it can be concluded that Nβs are released to an extracellular space and the cleavage site of the proteolysis immediately before the Nβ release is a novel fourth cleavage site (S4) that is different from the conventionally reported three cleavage sites (S1, S2, and S3).

[0037]

FIG. 4(b) shows a list of amino acid sequences of transmembrane domains of human (h) and murine (m) Notch1 to Notch4. S1, S2, and S3 cleavages are phenomena common to Notch1 to Notch4, and they serve as a common signal transduction mechanism through which Notch proteins, whatever their species, achieve signal transduction. From these facts, it is speculated that S4 cleavage also is a phenomenon common to Notch proteins of all types. As shown in FIG. 4b, the S4 cleavage site is conserved partially, similarly to the S3 cleavage site. From this fact, it is speculated that S4 cleavage is a phenomenon common to Notch1 to Notch4 proteins.

[0038]

(Example 4)

Confirmation of presenilin (PS) function dependence of extracellular release of Nβ

F-NEXT is stably transfected with cells expressing wild-type PS1 or PS1 D385N that is a PS1 dominant negative mutant obtained by artificially causing loss of presenilin function. An hour pulse with ($^{35}S$) and then a

2-hour chase were performed, and the resulting culture media and lysates were analyzed to determine an Nβ release level from the cells expressing both the PS1 derivative and F-NEXT at the same time. First, the chased media were immunoprecipitated with M2 agarose gel to detect Nβ release. As shown in the upper panel of FIG. 5a, Nβ release from the PS1 D385N expressing cells decreased drastically as compared with the case of the wild-type PS1 expressing cells. That is, the S4 cleavage efficiency decreases drastically in the cells expressing the mutant obtained by artificially causing loss of presenilin function. Also, the lysates collected at the same time with the culture media were immunoprecipitated with 9E10. As shown in the lower panel of FIG. 5a, NICD band after the 2-hour chase period was hardly visible in the PS1 D385N expressing cells. That is, the report that the S3 cleavage efficiency decreases drastically in the cells expressing the mutant obtained by artificially causing loss of presenilin function was reproduced at the same time.

[0039]

Next, cells stably expressing F-NEXT were pulse-labeled for 1 hour and chased for 2 hours with or without a γ-secretase inhibitor (L685,458) that is designed to bind the active center of presenilin. More specifically, 1 μM of L685,458 was added to the culture media 2 hours before methionine starvation. During the pulse-chase period, every medium used contained the same concentration of L685,458. The chased media were immunoprecipitated with M2 to detect Nβ release. As shown in the upper panel of FIG. 5b, Nβ release from the cells treated with the γ-secretase inhibitor decreased drastically. Also, the corresponding lysates were immunoprecipitated with 9E10. As shown in the lower panel of FIG. 5b, the NICD band after the 2-hour chase period was hardly visible due to

inhibition of S3 cleavage. From these results, it can be said that the Nβ release to an extracellular space is caused by presenilin-dependent proteolysis, and hence, inhibition of the presenilin function results in the inhibition of S4 cleavage and Nβ release that occurs subsequent to the S4 cleavage.

[0040]

(Example 5)

Effect of presenilin (PS) mutant associated with familial Alzheimer's disease (FAD) upon S4 cleavage

Heretofore, various studies have been made on PS mutation associated with FAD, and an increase in Aβ 42 secretion has been confirmed in every type of FAD pathogenic PS mutant. In the present example, it was confirmed that PS dependent S4 proteolysis also relates to PS mutation associated with FAD.

[0041]

K293 cells expressing wild-type (wt) PS1 or PS1 mutants associated with FAD, namely, PS1 C92S, PS1 L166P, and PS1 L286V, were stably transfected with F-NEXT. Then, the culture media of the cells expressing PS1 derivatives and F-NEXT were analyzed by MALDI-TOF MS, in order to examine the change in C-termini of F-Nβs. As shown in FIG. 6a, in contrast to the cells expressing wild-type PS1, characteristic change in a proteolysis pattern of C-termini of Nβs was observed in the cells expressing PS1 mutations associated with FAD. In particular, the cells expressing the PS1 L166P mutation causing a significant increase in Aβ42 production demonstrated a tendency to elongate F-Nβ peptides, and an increase in the production of F-Nβ species (F-Nβ 1733 and F-Nβ 1735) that were longer than F-Nβ1731 by 2 and 4 amino acid residues, respectively, was confirmed

(see FIG. 6b). Furthermore, as shown in FIG. 6a, an increase in F-Nβ 1734 level was observed in the PS1 C92S cells, whereas an increase in F-Nβ 1735 level and a decrease in F-Nβ 1734 level were observed in the PS1 L286V cells. These results demonstrate that FAD pathogenic mutations affects a pattern of the S4 cleavage site so that the S4 cleavage site tends to shift toward the C-terminal side, thereby causing elongation of released peptides. Similarly to Aβ42, the aggressive PS1 L166P pathogenic mutation affects the length of F-Nβs most significantly. It has been known that PS1 L166P mutation causes FAD during the young adult years. These effects were not specific to K293 cells, and the similar effects of the PS pathogenic mutations associated with FAD also were confirmed when using Neuro 2a cells (data not shown). Therefore, it can be said that every type of FAD pathogenic mutation affects the C-terminus of F-Nβ (see FIG. 6c).

[0042]

[Effects of the Invention]

As specifically described above, a novel polypeptide according to the present invention is derived from a Notch protein. In a series of proteolytic events of the Notch protein, the polypeptide is released to an extracellular space when NICD translocates to a nucleus as a result of intramembranous endoproteolysis that occurs subsequent to extracellular proteolysis. By using the novel polypeptide as a marker, it is possible to detect Notch signal transduction. Also, it is possible to detect cell differentiation, cell tumorigensis, apoptosis, Alzheimer's disease, etc., for example.

[0043]

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[Brief Description of the Drawings]

[FIG. 1] FIG. 1a is a schematic illustration of structures of NΔE, F-NEXT, and NICD. FIGs. 1b to 1c are electrophoretograms showing an

example of the production of FLAG-tagged novel polypeptides (Nβs) according to the present invention.

[FIG. 2] FIG. 2a to 2b are electrophoretograms showing an example of the production of novel polypeptides (Nβs) according to the present invention.

[FIG. 3] FIG. 3a is a chart showing the result of mass spectroscopy with regard to a group of novel polypeptides according to the present invention. FIG. 3b shows a major site of a novel cleavage (S4) of a Notch protein and major cleavage sites of an Alzheimer's disease β-amyloid precursor protein (hβAPP).

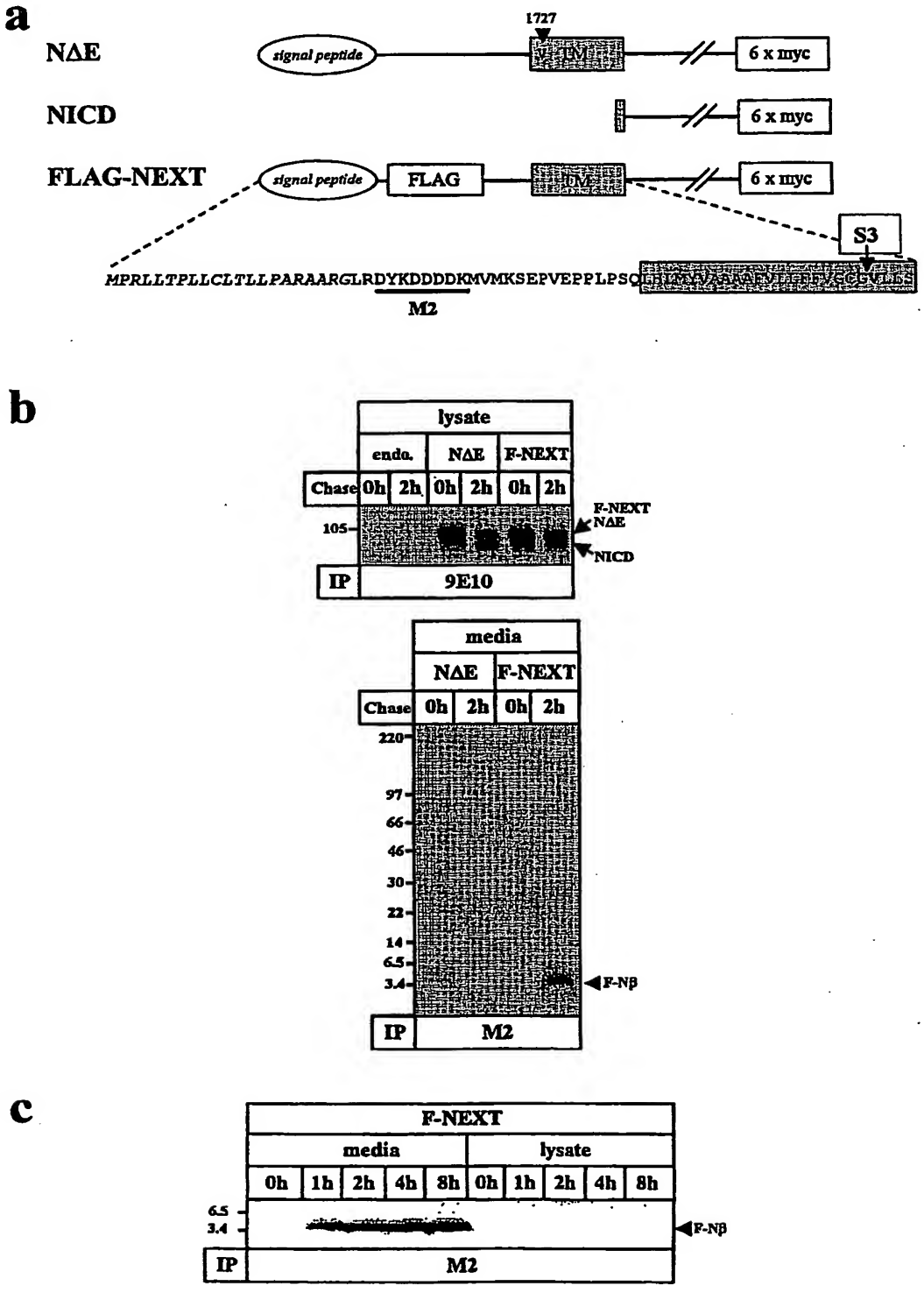
[FIG. 4] FIG. 4a is an example of amino acid sequences of the novel polypeptides as a principle part of the present invention. FIG. 4b is a view showing the comparison between intramembranous amino acid sequences of Notch1 to Notch4 and that of hβAPP.

[FIG. 5] FIGs. 5a, 5b are electrophoretograms showing an example of the effect of inhibition of presenilin (PS) function upon extracellular release of novel polypeptides (Nβs) according to the present invention.

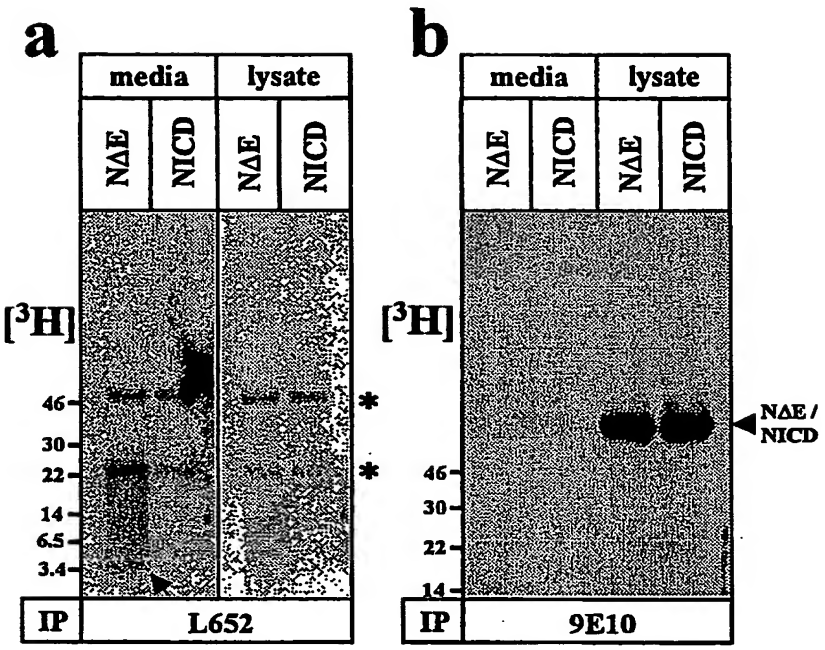
[FIG. 6] FIG. 6a is a chart showing the result of mass spectroscopy, which shows an example of the effect of Alzheimer's disease pathogenic presenilin mutants upon Nβ release. FIG. 6b shows Nβ species whose secretion is relatively increased by the effect of Alzheimer's disease pathogenic presenilin mutants. FIG. 6c shows the result of a semiquantitative analysis of the relative increase of their secretion.

[FIG. 7] A schematic illustration of an example of extracellular release of novel polypeptides (Nβs) according to the present invention and illustrates the C-terminus of the released peptide is changed by Alzheimer's disease pathogenic presenilin mutants.

[Document Name] Drawings
[FIG. 1]



[FIG. 2]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media
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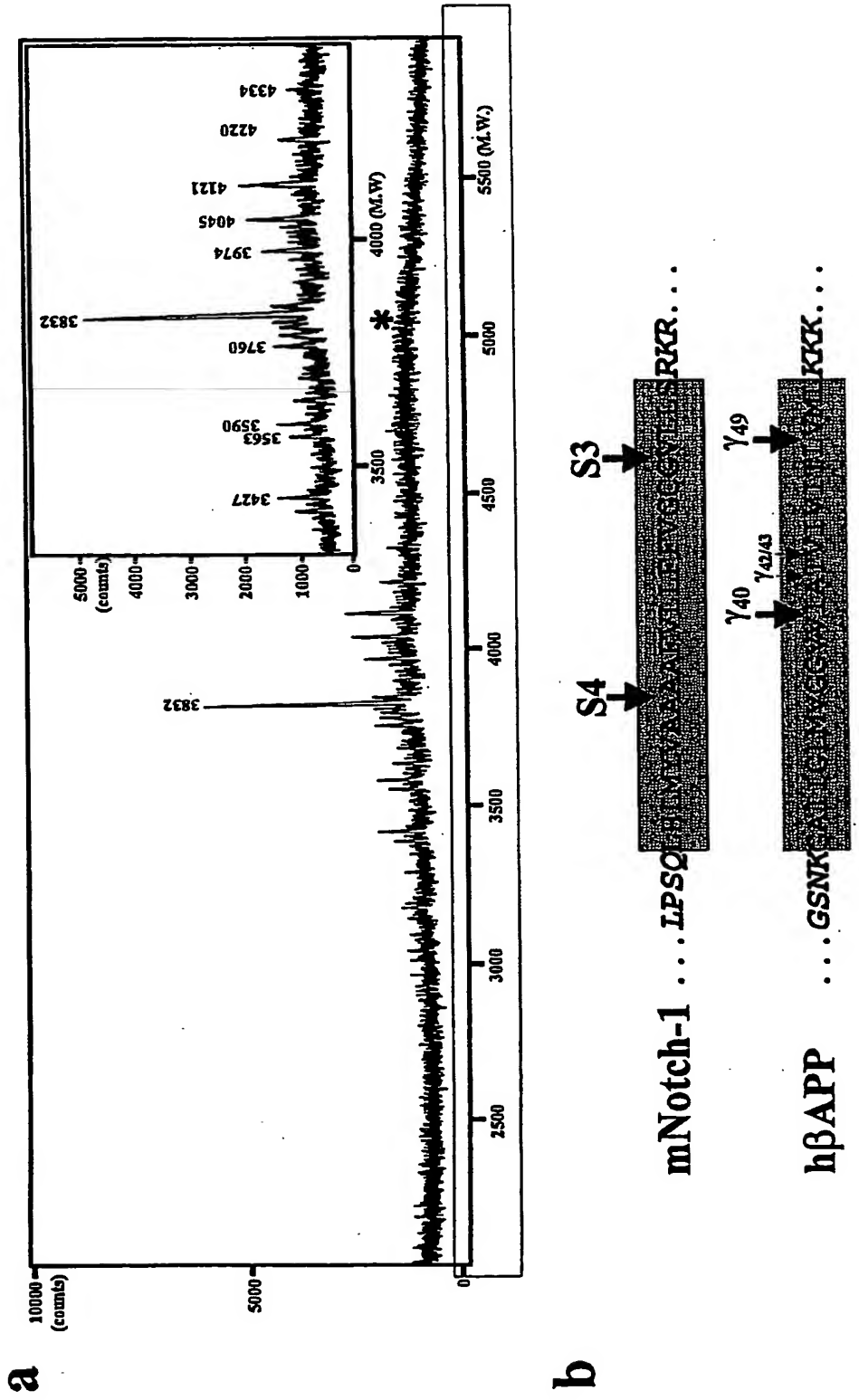
[FIG. 3]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b
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S3
S4
γ49
γ40
γ40/43
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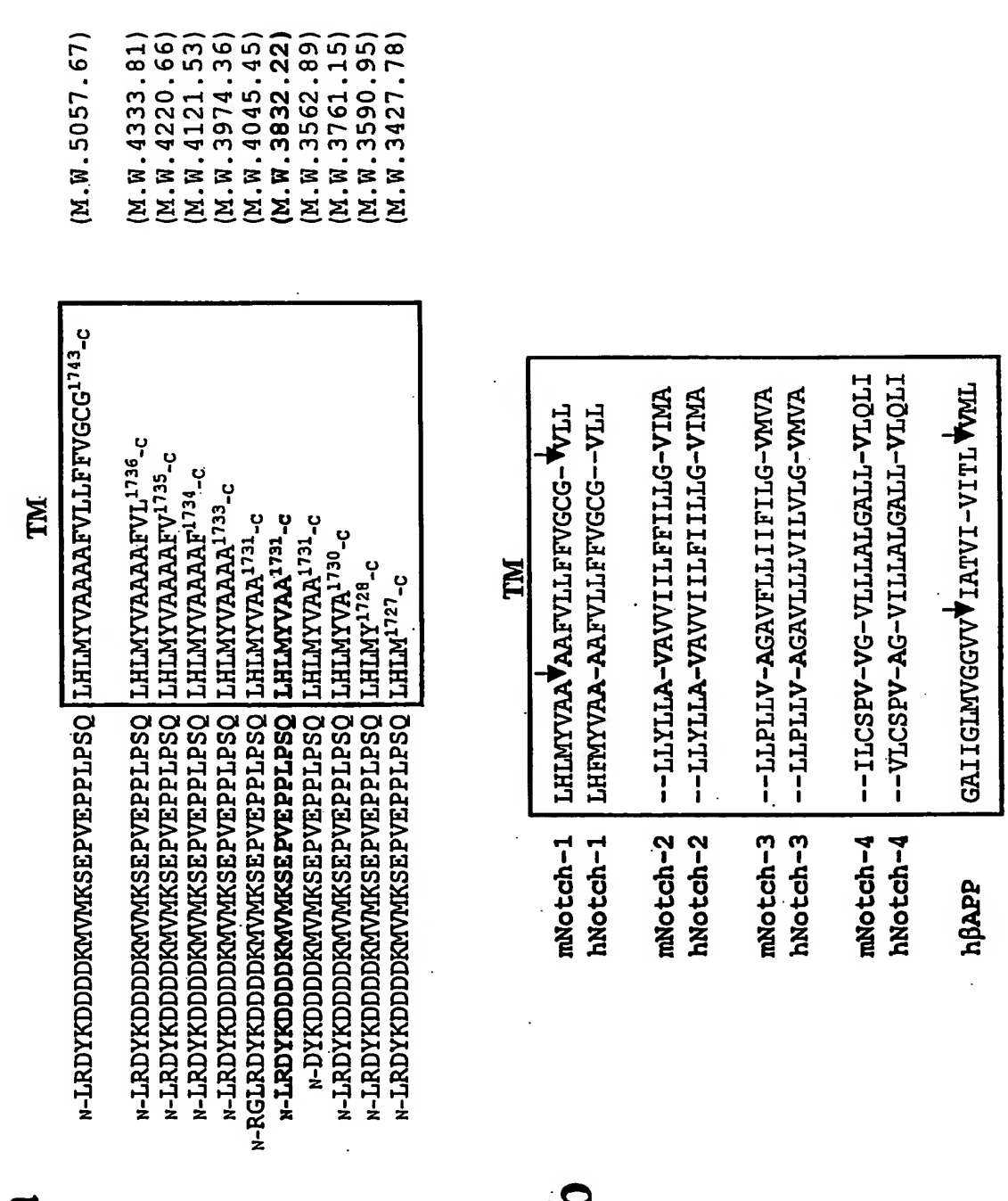
[FIG. 4]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hNotch-3 --LLPLLV-AGAVLLLVILVLG-VMVA
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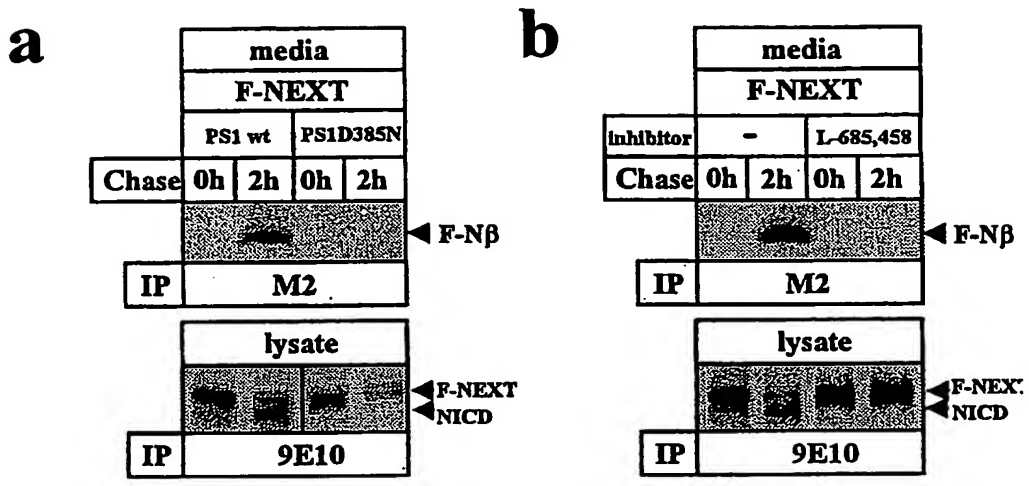
[FIG. 5]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2h
0h
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NICD
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9E10
b
media
F-NEXT
inhibitor
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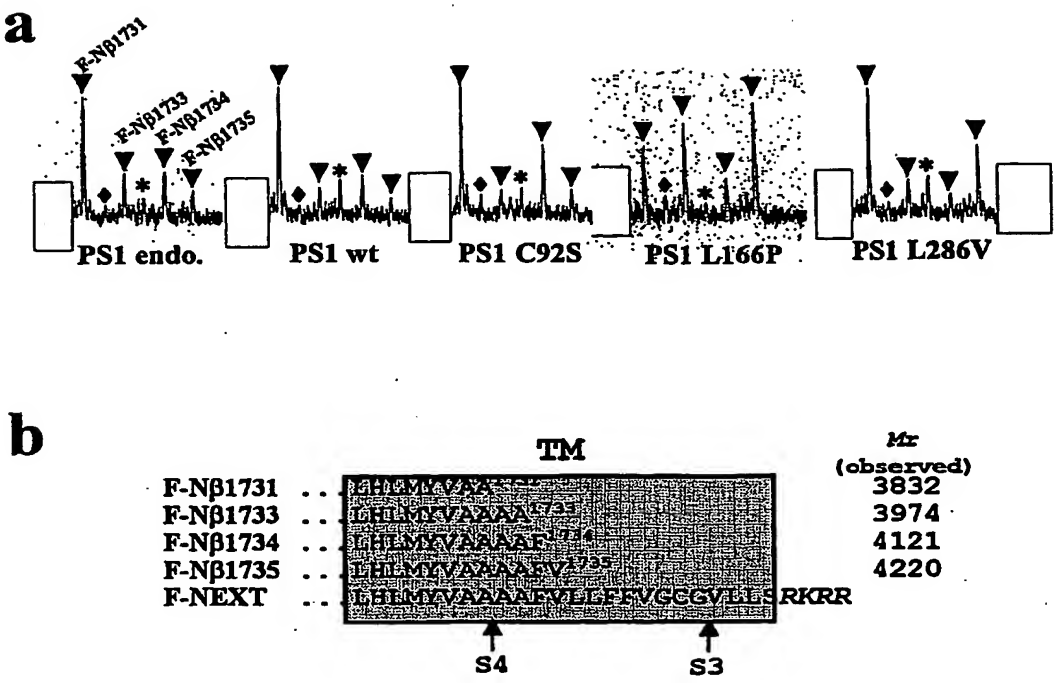
[FIG. 6]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F-Nβ1733
F-Nβ1734
F-Nβ1735
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PS1 wt
PS1 C92S
PS1 L166P
PS1 L286V
b
TM
Mr (observed)
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F-Nβ1733 3974
F-Nβ1734 4121
F-Nβ1735 4220
F-NEXT
RKRR
S4
S3
c
F-Nβ1733
F-Nβ1734
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PS1 wt
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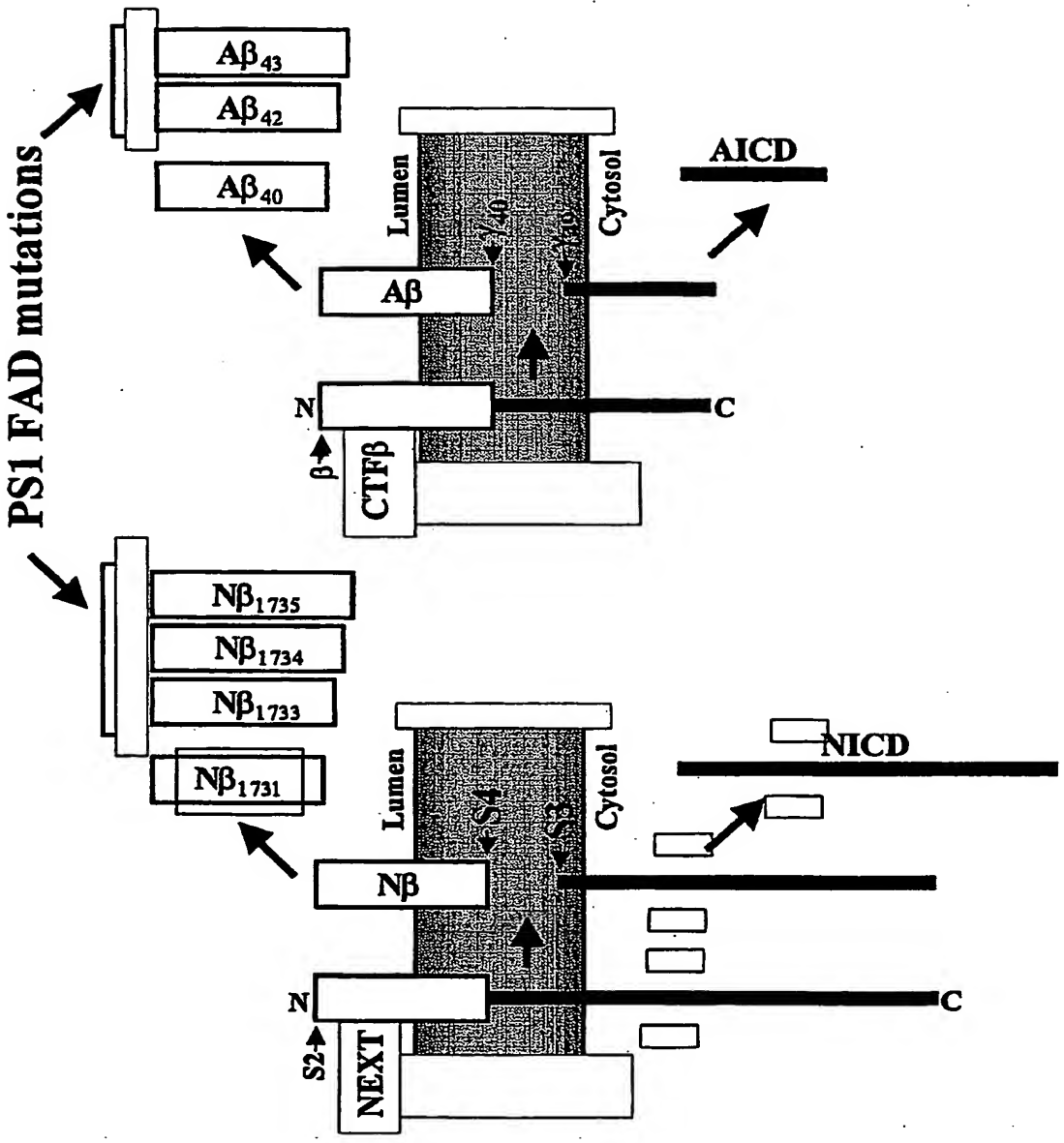
PS1 L166P
PS1 L286V

[FIG. 7]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Aβ42
Aβ40
Lumen
Cytosol
AICD
ε49
ε40
Aβ
N
C
β
CTFβ
Nβ1735
Nβ1734
Nβ1733
Nβ1731
NICD
S4
S3
Nβ
N
C
S2
NEXT

[Document Name] ABSTRACT

[Abstract]

[Objective] To provide an extracellular marker that can detect Notch signal transduction.

[Means for Solving the Problem] A group of novel polypeptides derived from Notch proteins wherein, in a series of proteolytic events of the Notch proteins, the group of polypeptides (Nβs) is released to an extracellular space when NICD (Notch intracellular cytoplasmic domain) translocates to a nucleus as a result of an intramembranous endoproteolysis that occurs subsequent to the extracellular proteolysis and used as a marker. The group of peptides (Nβs) that is released to the extracellular space in proportion to a Notch signal is presenilin dependent. The detection of the group of peptides allows for monitoring of Notch signal transduction, cell differentiation, cell tumorigensis, apoptosis, Alzheimer's disease, etc.

[Selected Figure] FIG. 7